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# Arterial Shear Stress Stimulates Surface Expression of the Endothelial Glycoprotein Ib Complex

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**Abstract** Exposure to shear stress has been shown to alter the expression of a number of surface components of cultured endothelial cells (EC). However, relatively few studies have examined the status of human EC surface proteins after prolonged flow, more closely corresponding to the steady state in vivo. Since the promoter region of glycoprotein (Gp) Ibα contains several copies of a putative shear stress response element, 5'-GAGACC-3', we investigated the response of cultured human umbilical vein EC (HUVEC) GpIbα to shear stress over a 72 h time period. In response to 30 dynes/cm$^2$ of shear stress, total cell content of GpIbα protein was markedly increased above static levels at 7 and 24 h, as determined immunohistochemically. Western blot analysis of whole cell lysates after 24, 48, and 72 h of shear treatment demonstrated a 2.4-, 4.1-, and 3.2-fold increase in total GpIbα protein, respectively. Cell surface protein expression of GpIbα increased 2.5-fold at 7 h, as measured by quantitative immunofluorescence, and remained at that level at 24 h. After 48 h of shear stress, cell surface GpIbα, GpIX, and GpV, analyzed by flow cytometric analysis, were further increased over the levels observed at 24 h. The increase in cell surface membrane expression of GPIbα at 24, 48, and 72 h was confirmed by immunoprecipitation of biotinylated surface proteins. No upregulation of GpIbα was noted after exposure to shear stress of 1–3 dynes/cm$^2$. These observations imply that under steady-state arterial shear conditions endothelial expression of the GpIb complex is significantly greater than observed in static EC cultures, and raise the possibility of a more important role for this complex under flow, rather than static conditions. J. Cell. Biochem. 73:508–520, 1999. 

**Key words:** blood vessels; hematology; vascular endothelium; gene expression; thrombosis

The endothelial cell lining of the blood vessel wall is continuously exposed to the forces generated by flowing blood, resulting in structural and functional characteristics different in many respects from those observed in static cultures [Davies, 1989; Dewey et al., 1981; Levesque and Nerem, 1985; Davies, 1995; Malek and Izumo, 1995; Gimbrone et al., 1997; Konstantopoulos and McIntire, 1996]. Study of cultured EC exposed to shear stress have demonstrated both early (seconds to minutes) and more delayed (hours to days) effects [Davies, 1989]. Early responses of cultured EC to the initiation of flow include the opening of ion channels, transient increases in intracellular $Ca^{2+}$ (1, 4), synthesis and release of $PGI_2$, [Davies, 1989; Dewey et al., 1981; Levesque and Nerem, 1985; Davies, 1995; Nollert et al., 1989; Nollert et al., 1992], elevation of $IP_3$ [Nollert et al., 1992; Prasad et al., 1993], and increased expression of the immediate early response genes fos and jun and the transcription factors NFκB and AP-1 [Nollert et al., 1992, Prasad et al., 1993, Lan et al., 1994, Knachigian et al., 1995]. Over a period of hours other endothelial cell genes are upregulated [Ando et al., 1996], including tPA [Diamond et al., 1989], PDGF-B [Resnick et al., 1993], TGF β-1 [Ohno et al., 1995], and ICAM-1, [Nagel et al., 1994], or down-regulated, of which endothelin-1 is an example

Abbreviations used: APAAP, alkaline phosphatase-anti-alkaline phosphatase; CHO, Chinese hamster ovary; EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; $IP_3$, inositol-1,4,5-trisphosphate; MCP-1, monocyte chemotactic protein-1; PAI-1, plasminogen activator inhibitor-1; PDGF-B, platelet-derived growth factor B; PGI2, prostacyclin; SSRE, shear stress response element; TGFβ-1, transforming growth factor β-1; TNF-α, tumor necrosis factor α; tPA, tissue plasminogen activator; VCAM-1, vascular cell adhesion molecule-1.

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[Malek and Izumo, 1995; Morigi et al., 1995]. Several genes, including c-fos, PDGF-A, c-myc, and c-jun [Malek and Izumo, 1995], respond to shear stress transiently; their expression returns to baseline within 1–4 h. Other genes, such as PAI-1 and ELAM-1 [Diamond et al., 1989; Nagel et al.,1994; Morigi et al., 1995], are not appreciably affected by shear stress. Shear also induces remodeling of the EC cytoskeleton, a process that can be detected within hours [Levesque and Nerem, 1985, Davies, 1995], but only reaches completion in HUVEC after 48–96 h. During this time the cytoskeleton reorients in the direction of flow, and EC change from a cuboidal to an ellipsoid morphology, concomitant with an increase in membrane rigidity [Davies, 1995; Dewey et al., 1981; Levesque and Nerem, 1985; Davies, 1995]. A hexanucleotide sequence, 5'-GAGACC-3', or its complement, has been identified within the PDGF-B promoter region that is required for the transcriptional upregulation of this gene in response to shear stress [Resnick et al., 1993]. This shear stress response element (SSRE) is present in the promoter region of a number of shear stress-responsive genes [Davies, 1995; Resnick et al., 1993; Nagel et al., 1994; Morigi et al., 1995]. However, other transcriptional elements, such as Egr-1, TRE, and Sp1 sites, have been implicated in the shear-induced increase, or in the case of the VCAM-1 promoter [Gimbrone et al., 1997], decrease, in endothelial gene expression [Davies, 1995; Shyy et al., 1995]. Thus, gene regulation by shear stress is likely to be a complex process.

Endothelial cells, like platelets, express all the components of the GpIb complex [Asch et al., 1988; Konkle et al., 1990; Kroll et al., 1996; Rajagopalan et al., 1992; Sprandio et al., 1988; Wu et al., 1997]. The presence of seven copies of the SSRE in the GpIbα gene promoter region raises the possibility that shear stress may affect GpIbα gene expression. We report here that exposure of EC to arterial shear stress induces a substantial increase in surface expression of the EC GpIb complex, an increase which is maintained for at least 72 h, the longest period of shear stress we examined.

## MATERIALS AND METHODS

### Cell Culture

HUVEC were isolated from umbilical veins and cultured as described [Sprandio et al., 1988]. After reaching confluence in a T-75 tissue culture flask, HUVEC were dissociated with trypsin/EDTA for 30 sec, and resuspended in complete medium (Medium 199, 20% bovine serum, 100 μg/ml sodium heparin and endothelial cell growth factor) before centrifugation at $300 \times g$ for 5 min, as previously described [Beacham et al., 1992; Beacham et al., 1995; Beacham et al., 1997].

For shear studies, 7.5 × 5 cm glass slides (Corning Glass, Vineland, NJ) were pretreated with 0.5 M NaOH for 2 h, washed briefly with nanopure $H_2O$ and autoclaved. A hydroscopic marking pen, the PAP pen (Research Products International Corp., Mt. Prospect, IL), provided a thin film-like barrier to define the cell surface plating area, 5.75 × 3.35 cm (total area 19.3 $cm^2$), after which the slides were exposed to UV light for 10 min to sterilize the PAP pen fluid. Under sterile conditions, slides were then immersed in a 1% gelatin solution at 37°C for 1 h. After draining the excess solution, the gelatin-coated slides were treated with a 100 μg/ml fibronectin solution at 37°C for 1 h. After the excess fibronectin was removed, first passage HUVEC from 2–3 T-75s were seeded onto the plating surface of the glass slide at a density of $5 \times 10^4$ cells/$cm^2$ and allowed to attach and spread for 2–3 h; slides were then flooded with complete medium. HUVEC were grown for 1–3 days, to reach confluence, prior to insertion in the flow apparatus. To evaluate EC GpIbα gene expression under static conditions, identical slides of first passage HUVEC were cultured simultaneously, as described above. Initially, HUVEC exposed to a low shear stress of 3 dynes/$cm^2$ were evaluated for GP Ibα expression by immunohistochemical staining, cell surface immunofluorescence, and Western blotting. GP Ibα expression under low shear stress conditions was indistinguishable from GP Ibα expression on HUVEC grown in the tissue culture incubator in a 37°C, 5% $CO_2$ atmosphere, grown on either glass slides or in T-75 tissue culture flasks. Since no difference was observed between EC cultured under low shear stress or grown in the $CO_2$ incubator, slides or T-75 tissue culture flasks grown in the incubator were used as static HUVEC samples in this study.

### Flow Apparatus

Confluent endothelial cells were exposed to a shear stress of 30 dynes/$cm^2$ using a parallel

plate flow chamber with a gap height of 100 μm, modified from the design of McIntire and coworkers [Hubbell and McIntire, 1986; Konstantopoulos and McIntire, 1996; Wick et al., 1987]. Shear stress was calculated by determining the flow rate of medium emitting from the outlet port of the flow chamber as a measure of the flow rate through the flow chamber. The HUVEC-coated slides formed the base of the flow chamber and were held in place on the assembled chamber with an applied vacuum [Wick et al., 1987]. A continuous circuit was utilized in these studies, consisting of the flow chamber mounted on a dry bath incubator (Fisher Scientific, Pittsburgh, PA) connected with silastic tubing to a Rainin Dynamax model RP-1 peristaltic pump, chosen based on its smooth flow rate and minimal pulsatile flow. EC plated in the flow chamber were exposed to constant laminar flow with medium directly supplied by a reservoir bottle placed between the pump and the chamber to dampen pulsation from the pump. The flow system was adjusted to 37°C and continuously perfused with humidified $CO_2$, modified from the design of Nollert and coworkers [Nollert et al., 1989]. Under these conditions the HUVEC monolayer, cultured in complete medium containing 20% bovine serum with supplemental iron, could be maintained intact for at least 72 h, the longest time period studied in these experiments. Endothelial cells were visualized and recorded using a Nikon Diaphot microscope and Hamamatsu C2400-77 CCD camera system (Optical Apparatus Co., Ardmore, PA). HUVEC cultured under static conditions or exposed to a shear stress of 30 dynes/cm$^2$ for 7–72 h were dissociated with trypsin/EDTA to determine cell number, as described [Beacham et al., 1995; Beacham et al., 1997; Beacham et al., 1992]. Equivalent numbers of HUVEC, about $1.5 \times 10^6$ cells/slide, were harvested from confluent static or shear stress-exposed HUVEC. A cell viability of about 90% was obtained from both static and shear-exposed slides using trypan blue dye exclusion. Total protein content from this number of cells was determined to be approximately 150 μg from both static and shear-exposed slides, based on the BCA protein assay (Pierce Chemical Co., Rockford, IL). For flow cytometric or Western blot analysis of HUVEC proteins under shear stress conditions, larger flow chambers were constructed (William Pennie, Research Instrumentation Group, University of PA) modified from the design of Nerem and coworkers [Prasad et al., 1993]. The large flow chambers provide a 6.2 × 12.5 cm flow area (total area 77.5 cm$^2$). The gap height for these larger chambers is 100 μm, identical to the gap height of the smaller chambers. HUVEC were plated onto gelatin-coated rectangular slides of tissue culture plastic and grown to confluence for 1–3 days prior to exposure to shear stress, as described above for glass slides. The larger flow chambers yielded 5–7 × $10^6$ cells/chamber, sufficient for 8–10 flow cytometric analyses.

### Immunohistochemistry

Confluent HUVEC exposed to shear stress or cultured under static conditions were stained by the APAAP method, as previously described [Konkle et al., 1990; Rajagopalan et al., 1992; Cordell et al., 1984]. Endothelial cells were fixed and permeabilized by incubation with acetone for 10 min at room temperature and air-dried. HUVEC were incubated overnight with a 1:50 dilution of nonimmune rabbit serum in TBS (Tris-buffered saline: 50 mM Tris, 150 mM NaCl, pH 7.6) prior to incubation with the murine anti-GpIbα monoclonal antibodies (mAbs) 1b1 [DeMarco et al., 1991](kindly provided by Dr. Z. Ruggeri, Scripps Research Institute, La Jolla, CA) or AS-7 [Miller et al., 1990] (kindly provided by Dr. Jonathan Miller, Health Science Center at Syracuse, Syracuse, NY) or with normal mouse IgG. For comparison, HUVEC were also incubated with rabbit polyclonal antibody to human PAI-1 (kindly provided by Dr. David Ginsburg, University of Michigan, Ann Arbor, MI) for 30 min followed by mouse anti-rabbit IgG (Dako Corp., Carpinteria, CA). All antibodies were diluted 1:50 in TBS containing 0.2% normal goat serum (Sigma, St. Louis, MO) to further reduce nonspecific antibody binding. After incubation with mouse monoclonal primary antibodies or mouse anti-rabbit IgG (PAI-1 only), HUVEC monolayers were incubated with rabbit anti-mouse IgG for 30 min. After washing with TBS, HUVEC were incubated with the APAAP complex (Dako Corporation) for 30 min and washed with TBS; the rabbit anti-mouse and APAAP incubations were repeated once more for 20, rather than 30, min. EC were

washed with TBS and incubated with the APAAP substrate for 15–20 min, washed extensively with nanopure $H_2O$ and counterstained with hematoxylin to visualize cell nuclei.

### Immunofluorescence

HUVEC exposed to shear stress were fixed in situ by perfusion for 30 min with 2% paraformaldehyde-PBS containing 1 mM $MgCl_2$, removed from the flow apparatus and washed with PBS alone. HUVEC cultured under static conditions were incubated for 30 min with the same fixative and washed with PBS prior to antibody staining. HUVEC cultured under either static or shear stress conditions were incubated with 1:100 dilutions of anti-GpIbα mAb 1b1 ascites, rabbit antiserum to PAI-1, a mAb to human vWF (DAKO Labs), or, as controls, a 1:100 dilution of normal rabbit serum or 10 μg/ml normal mouse IgG. Stock antibody solutions were diluted in PBS/0.1% BSA. For intracellular staining of vWF, HUVEC were permeabilized with PBS/0.5% Triton X-100 for 10 minutes prior to incubation with the vWF mAb. Following incubation with the primary antibodies, HUVEC were washed with PBS/0.1% BSA and incubated at room temperature for 1 h either with a 1:1,000 dilution of goat anti-mouse IgG Texas Red conjugate (GAM-Texas Red, Molecular Probes Inc., Eugene, OR), for anti-vWF, or with goat anti-rabbit IgG Texas Red (Molecular Probes Inc.), for anti-PAI-1.

For immunofluorescence quantitation, a 1:1,000 dilution of goat anti-mouse IgG fluorescein isothiocyanate (FITC) was used as secondary antibody following incubation with mAb 1b1. Following staining, endothelial cells were washed in PBS for 5 min and mounted with a 1:1 mixture of PBS/glycerol containing n-propyl gallate, to prevent fluorescence bleaching (Slowfade, Molecular Probes, Inc.). HUVEC were photographed with Kodak Ektapress 1600 film using a Nikon Labophot-2A EF-D-PH microscope equipped with epifluorescence optics. Fluorescence intensity was quantified with NIH Image Analysis software on a Power Macintosh 7100/66 system. Individual endothelial cells from static and shear-exposed samples were digitized from captured images of several representative fields at a final magnification of 60×. The fluorescence intensity of each cell in a field was quantified in units of density/$\mu m^2$ to normalize fluorescence between cells of slightly different shapes. The error bars represent the standard deviation calculated from 75–100 cells.

### Flow Cytometry

Static or shear-treated HUVEC were dissociated with lifting buffer ($Ca^{2+}$- and $Mg^{2+}$-free PBS (GIBCO BRL, Gaithersburg, MD), supplemented with 24 mM $NaHCO_3$, 0.2 mg/ml BSA, 10 mM Hepes, 10 mM EDTA, 5 mM EGTA, and 1 mM PMSF) and washed twice in PBS. Both static and shear stress-treated HUVEC, about $8–10 \times 10^6$, were resuspended in 1 ml of PBS. After washing twice with PBS and once with 0.2% BSA-PBS, cells were aliquoted into 1.5 ml sterile Eppendorf tubes for staining with primary antibodies. Cells were stained for the GpIbα, GpIX, and GpV subunits of the GpIb complex with mAbs 1b1, FMC-25, and 1D9 (generous gift of Dr. Toshiro Takafuta, Cardeza Foundation), respectively. Endothelial cells were also stained for GpIbα with a polyclonal antibody to glycocalicin (poly GC), prepared in our laboratory [Wu et al., 1997]. A mAb, 10E2, directed against the $\beta_3$ integrin subunit, was prepared in our laboratory by Dr. Guoxin Wu [Beacham et al., 1997] and used as a control. HUVEC were incubated for 1 h at room temperature with a 1:50 dilution of the appropriate monoclonal or polyclonal antibodies in 1% BSA/PBS. Cells were washed twice with 0.2% BSA-PBS and fixed with 1% paraformaldehyde in PBS for 1 h at room temperature. HUVEC were washed twice with 0.2% BSA-PBS and stained with goat anti-mouse IgG fluorescein isothiocyanate (GAM-FITC) or goat anti-rabbit IgG FITC (GAR-FITC) at a 1:10 dilution in 1% BSA/PBS. Cells were counted and analyzed with a Coulter flow cytometer interfaced with an EPICS profile analyzer. Data were expressed as particle (cell) number vs. log fluorescence intensity over a log scale ranging from 0.1–1,000. To determine whether we were detecting intracellular staining with the standard fixation procedure described above, parallel aliquots of HUVEC were either fixed or fixed and permeabilized with a two-step fix and permeabilization kit (Caltag Labs, Inc., Burlingame, CA) and then stained for actin-binding protein 280 (ABP-280) with the mononclonal antibody Mab-1 (generous gift of Dr. John Hartwig, Brigham and

Women's Hospital, Boston, MA), as described above.

### Chemiluminescence Western Blotting

HUVEC whole cell lysates were prepared by lysing EC in situ on either the flow chamber slide for shear-treated HUVEC, or the T-75 tissue culture flask for static cultured HUVEC with lysis buffer (10 mM Tris-HCl, 0.5% deoxycholate, 0.5 % Triton X-100, 1 mM $Na_2EDTA$, 150 mM NaCl, pH 7.5) to which protease inhibitors (5 mM benzamidine, 5 mM EDTA and 1 mg/ml aminoethylbenzenesulfonylfluoride, AEBSF) were added. Insoluble debris was pelleted at 10,000 rpm in an Eppendorf microfuge and the protein content of the supernatant was determined colorimetrically with the standard BCA protein assay kit (Pierce Chemical Co., Rockford IL). Equal amounts of static and shear-treated HUVEC lysate, from 5–15 µg, were separated on a 10% SDS-PAGE gel under reducing or non-reducing conditions and then electroblotted onto a PVDF membrane (Dupont-NEN) in transfer buffer (50 mM Tris, 195 mM glycine, 12% methanol and 0.015% SDS, pH = 8.3). After transfer, non-specific binding sites were blocked with blocking buffer (50 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.05% Tween-20, pH 7.2) with added 2% nonfat Carnation dry milk for 1 h. The membrane was washed twice for 5 min prior to incubation with primary antibodies (rabbit polyclonal anti-GC, 1:200 or nonimmune rabbit IgG, 1:200) diluted in 1% BSA (98% pure, crystallized BSA from ICN Pharm., Costa Mesa, CA) in blocking buffer for one hour with shaking. The primary antibody solution was decanted and the membrane washed once for 15 min, then four times for 5 min intervals in blocking buffer. The blot was incubated in a 1:5,000 dilution of goat anti-rabbit IgG-horse radish peroxidase (GAR-HRP) in 1% BSA/blocking buffer for one hour with shaking. The membrane was washed once for 15 min followed by four 5 min washes, before transferring the blot to blocking buffer without Tween-20 for another four, 5 min washes. The blot was developed in Renaissance chemiluminescence reagent Plus (DuPont-NEN) and exposed to X-ray film (Labscientific Inc., Livingston, NJ). Protein bands were quantified with NIH Image software version 1.61b8 on a Power Macintosh 7600/120 system.

### Biotinylation of HUVEC Surface Proteins and Avidin-HRP Western Blotting

Confluent monolayers of static or shear-treated HUVEC were washed three times with ice-cold PBS (NaCl, 8 g/l, KCl, 2 g/l, $Na_2HPO_4$ 1.15 g/l, and $KH_2PO_4$, 0.2 g/l, pH 8.0). PBS was gently removed and cultures were biotinylated by incubation with a 0.5 mg/ml solution of sulfo-NHS-biotin (Pierce Chemical Co.) in PBS, pH 8.0, for 30 min at room temperature with shaking, as described [Altin and Pagler, 1995]. Biotinylated HUVEC were washed three times with ice-cold PBS, pH 8.0, and solubilized in lysis buffer with added protease inhibitors as described for Western blotting. Endothelial GP Ibα was immunoprecipitated from static and shear-treated EC lysates with the rabbit polyclonal GC antibody, or with nonimmune rabbit IgG, as previously described [Beacham et al., 1997]. Static or shear lysates, 1 ml, were incubated overnight with 10 µg/ml primary Ab at 4°C with shaking; the following day 20 µl of protein A-Sepharose CL-4B beads were added and incubated for 1 h at 4°C. The immune complexes were pelleted for 1 min at 200 × g, washed four times with lysis buffer, and once with TBS (Tris buffered saline: 50 mM Tris, 150 mM NaCl, pH 7.4) before being released by incubation at 70°C for 5 min in reducing sample buffer. Samples were run on a 10% SDS-PAGE gel and labeled proteins were detected with a 1:20,000 dilution of avidin-HRP (Pierce Chemical Co.) diluted in 0.1% BSA/PBS, pH 7.5, as described by the manufacturer. Blots were developed with the Renaissance chemiluminescence reagent, as described above.

## RESULTS

As shown in Figure 1A, total cellular GpIbα protein expression was increased by shear, an effect noted after 7 h (data not shown), and still obvious after 24 h of shear (Fig. 1A). In contrast, exposure of HUVEC to shear stress did not increase cell-associated protein expression of PAI-1 (Fig. 1B). Increased PAI-1 as well as GpIbα protein expression under static conditions was easily demonstrated after TNF-α treatment (data not shown), as we, and others have reported [Rajagopalan et al. 1992; Van den Berg et al., 1988]. Total protein expression of endothelial GP Ibα was also determined by

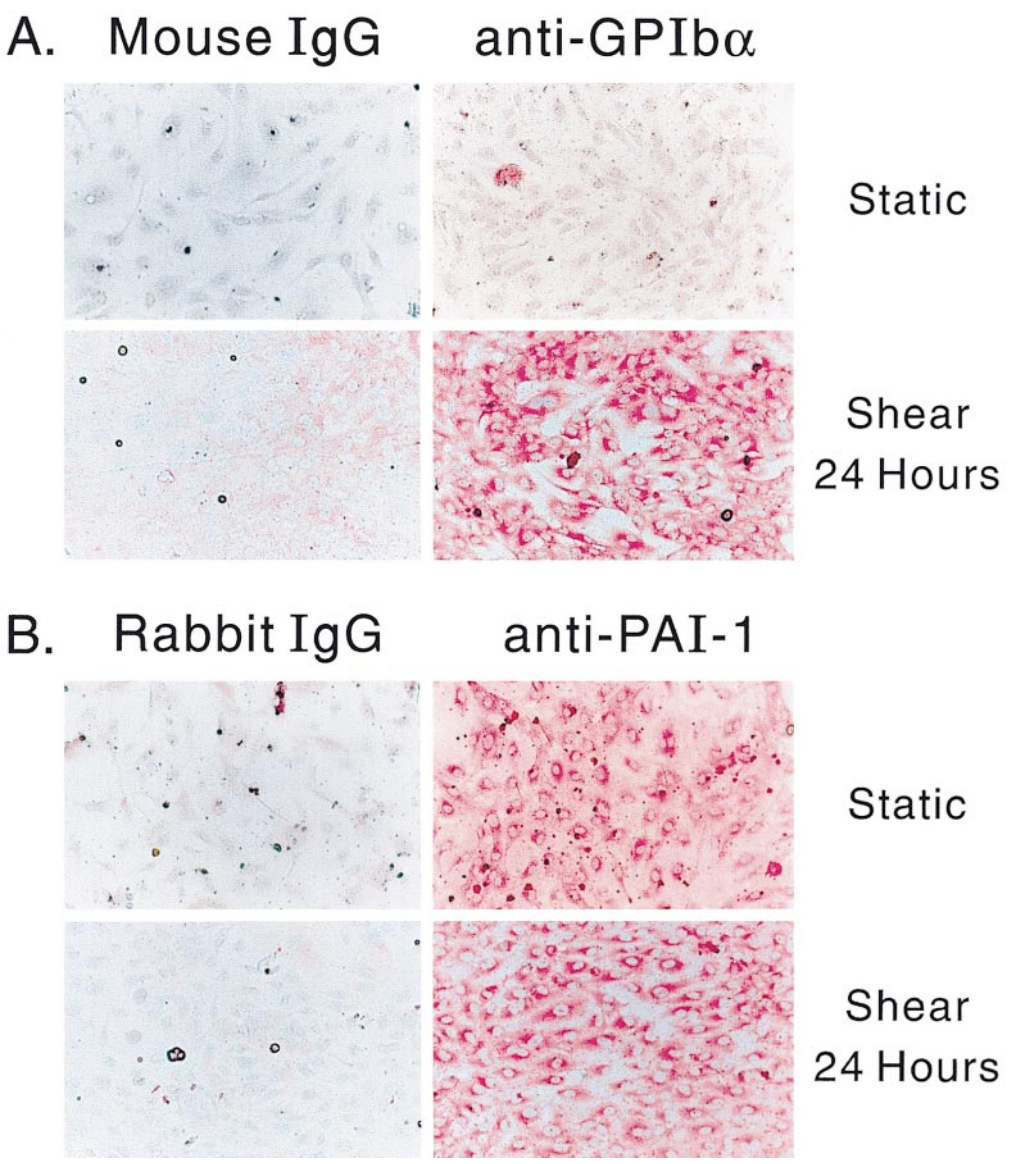


**Fig. 1.** **A:** Immunohistochemical staining for EC GpIbα under static and shear stress conditions. HUVEC cultures were stained by the APAAP method with either mouse IgG (left panels) or the anti-GpIbα Mab 1b1 (right panels). The upper panel depicts HUVEC grown under static conditions; the lower panel depicts HUVEC exposed to a shear rate of 30 dynes/cm$^2$ for 24 h. **B:** Immunohistochemical staining of PAI-1 under static and shear stress conditions. HUVEC cultures were stained by the APAAP method with either rabbit IgG (left panels) or the rabbit anti-PAI-1 antibody (right panels), under static conditions (upper panel) or after exposure to a shear stress rate of 30 dynes/cm$^2$ for 24 h (lower panel).

chemiluminescence Western blot analysis of whole cell lysates of HUVEC (Fig. 2). A band of Mr 150 kDa reacted with the poly GC antibody under reducing conditions in Western blot analysis. The 150 kDa band could be detected in static HUVEC lysates (lane 1), but after 24 h of exposure to a shear stress of 30 dynes/cm2 the shear/static ratio for this band was 2.4-fold increased (lane 2). At 48 h, the GP Ibα band in shear-exposed HUVEC (lane 4) was increased 4.1-fold relative to static levels (lane 3), and at 72 h, the shear/static ratio for the 150 kDa band remained increased above static levels by 3.2-fold (lane 5 vs. lane 6). A similar increase was observed in the 190 kDa GP Ibα band [Wu et al., 1997] detected under nonreducing conditions (data not shown).

We examined the effect of shear on the EC GpIbα surface expression in three ways. Using immunofluorescent staining of non-permeabi-

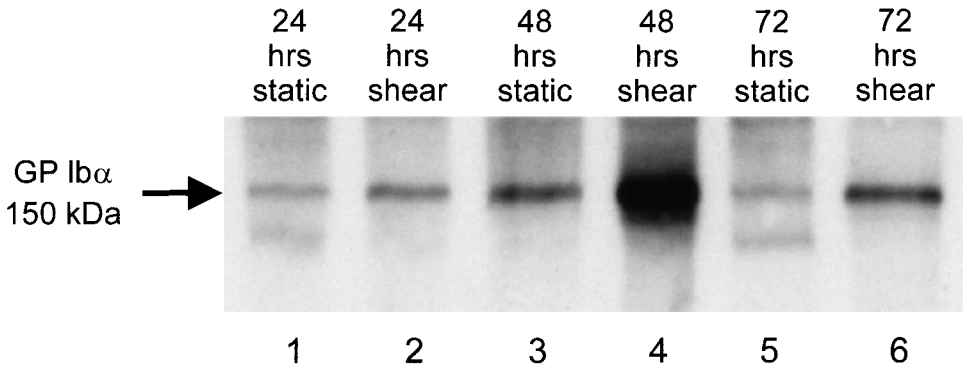


**Fig. 2.** Western blot analysis of static and shear-exposed HUVEC lysates with the rabbit polyclonal antibody to GC. Shear exposed and static HUVEC cultures were lysed and 15 μg of whole cell lysate was separated on 10% SDS-PAGE gels under reducing conditions and analyzed by chemiluminescence Western blotting with the GC antibody as described. Static lysate (**lane 1**) prepared from stationary HUVEC cultures or from EC exposed to shear for 24 h (**lane 2**) or 48 h (static, **lane 3**, shear, **lane 4**) were run side-by-side, as was lysate derived from static HUVEC (**lane 5**) or HUVEC cultures exposed to 72 h of shear (**lane 6**). The 150 kDa GP Ibα band is denoted with an arrowhead.

lized endothelial cells (Fig. 3), surface GpIbα was increased after 7 h of shear stress (panel I, A vs. B), and remained increased after a 24 h exposure to a shear stress of 30 dynes/cm$^2$ (panel II, A vs. B). Cell surface-associated PAI-1 staining, presumably due to the binding of secreted PAI-1 to vitronectin adsorbed to the culture dish surface [Stefansson and Lawrence, 1996], was not increased by exposure to 7 h of shear stress (panel I, E vs. F). By fluorescence quantitation (Fig. 4), surface GpIbα increased about 2.5-fold after 7 h, whereas no change was seen in surface-associated PAI-1. This increase was found to be highly significant statistically ($P$ = 0.03) using the Student's two-tailed $t$-test. In contrast, surface expression of PAI-1 was not altered by exposure to shear ($P$ = 0.24 for static compared with shear). Lack of permeabilization during paraformaldehyde fixation was demonstrated by the lack of staining for vWF in paraformaldehyde-fixed cells, whereas Triton-permeabilized cells showed strong staining (data not shown).

Cell surface protein expression of GpIbα, GpIX, and GpV was also evaluated by flow cytometry. As shown in Figure 5, an increase in GpIbα, a slight increase in GpIX, and no detectable change in GpV surface expression was observed in HUVEC exposed to 30 dynes/cm$^2$ shear stress for 24 h. After 48 h of shear stress a more substantial increase in staining on the endothelial cell surface was observed for GpIbα, IX and V (Fig. 5). The asymmetry of the peaks suggest a nonuniform response of the HUVEC to shear, with the cells displaying a broad range of increased expression for GP Ibα, IX, and V. To confirm these observations, HUVEC were stained with the rabbit polyclonal antibody to GPIbα, poly GC. The shear-induced increase in GpIbα staining was also evident with this antibody at both 24 and 48 h (Fig. 6). Similar to the mAb staining, staining of GP Ibα with poly GC was markedly increased at 48, compared with 24 h. The specificity of this is illustrated by our observation that the EC vitronectin receptor ($\alpha_v\beta_3$), as measured with the anti-$\beta_3$ mAb 10E2, was not changed by exposure of EC to 30 dynes/cm$^2$ of shear stress (Fig. 5, bottom panel). In order to verify that EC exposure to shear stress did not disrupt the cell membrane or render the cell permeable to the antibodies used for cell surface staining, both static and shear-exposed HUVEC were dissociated as described in Materials and Methods and stained with Mab-1, directed to the intracellular protein ABP-280, which links GpIbα to the actin cytoskeleton. Neither static nor shear-exposed HUVEC

**Fig. 3.** Cell surface immunofluorescence of EC GpIbα and PAI-1 under static and shear stress conditions. HUVEC monolayers were cultured under static conditions (left) or exposed to a shear stress of 30 dynes/cm$^2$ (right) for 7 h (Panel I) or 24 h (Panel II). Panel I: HUVEC were stained after 7 h with the GpIbα mAb, 1b1 (**A,B**), nonimmune mouse IgG (**C,D**), rabbit anti-PAI-1 (**E,F**), or nonimmune rabbit IgG (**G,H**) under static or shear conditions; final magnification: 69×. Panel II: HUVEC were stained after 24 h with the GpIbα mAb, Ib1 (A,B) or with nonimmune mouse IgG (C,D) under static (left) or shear (right) conditions; final magnification: 73×.

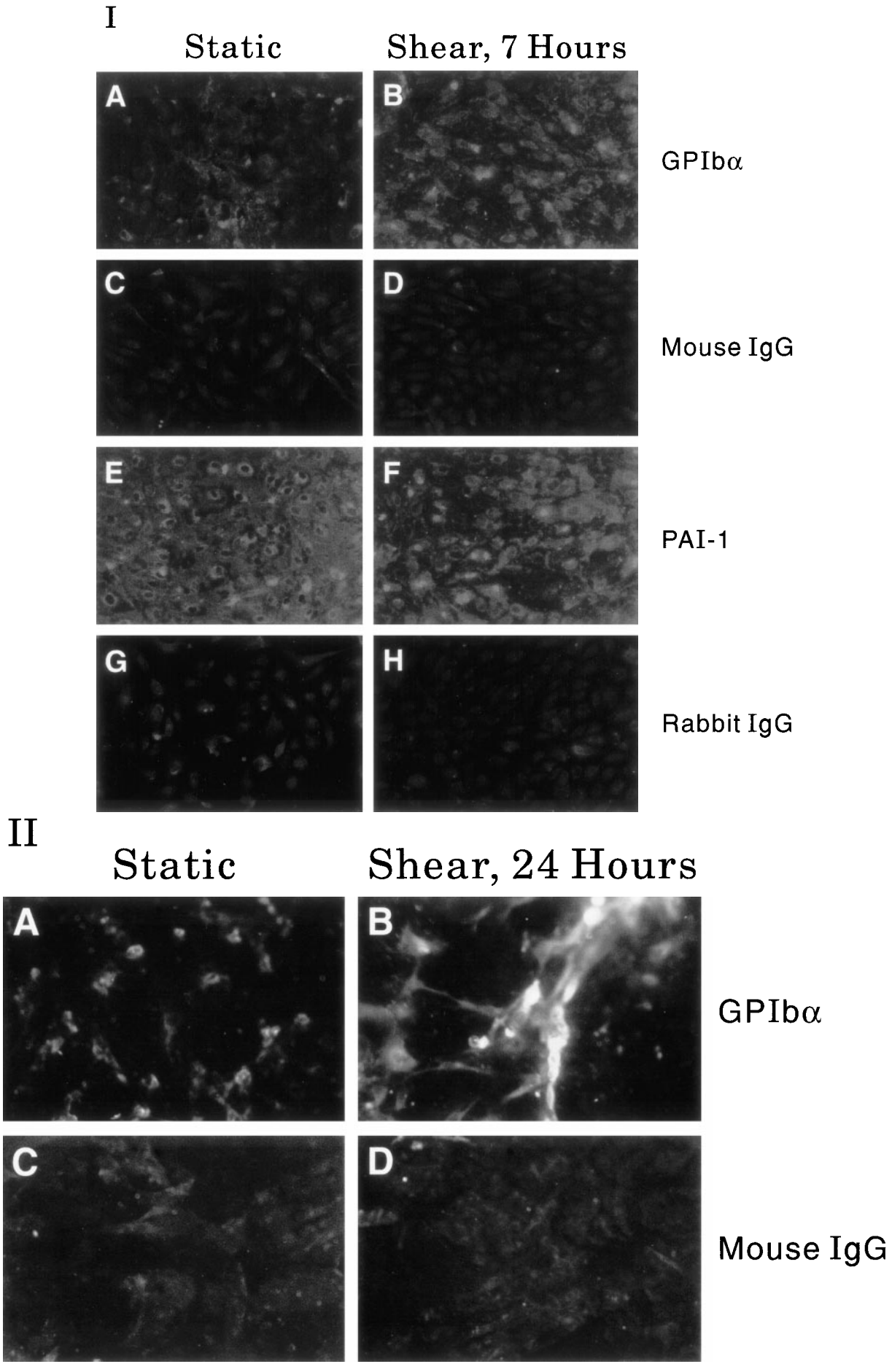
I
Static
Shear, 7 Hours
A
B
GPIbα
C
D
Mouse IgG
E
F
PAI-1
G
H
Rabbit IgG
II
Static
Shear, 24 Hours
A
B
GPIbα
C
D
Mouse IgG

Figure 3.

stained with this mAb. On the other hand, after permeabilization HUVEC stained strongly with this antibody (data not shown). Thus, the staining observed in these experiments reflects the binding of antibodies to the cell surface, and neither the dissociation procedure nor the exposure of EC to shear stress resulted in detectable permeabilization to antibodies.

As an additional approach, we biotinylated surface membrane proteins, lysed the cells, immunoprecipitated GP Ibα with the anti-GC polyclonal antibody, and detected immunoprecipitated proteins with avidin-HRP. As shown in Figure 7, GP Ibα was increased in shear-treated, compared with static HUVEC at all times examined. The two highest molecular weight bands most likely correspond to intact GP Ibα and glycocalicin, since the lower glycocalicin band yielded an estimated Mr = 150 kDa on static and shear-treated HUVEC. The shear/static ratio of the GP Ibα 150 kDa band was 2.3, 3.6, and 2.2 at 24, 48, and 72 h, respectively. Two other major bands of Mr 55 and 25 kDa, may correspond to EC GP Ibβ (55 kDa) and GPIX (25 kDa), respectively. The Mr = 80 kDa band is probably a proteolytic breakdown fragment of GP Ibα, as a band of this size was seen in previous immunoprecipitation studies [Beacham et al., 1997]. No bands were detected in the rabbit IgG control (data not shown).

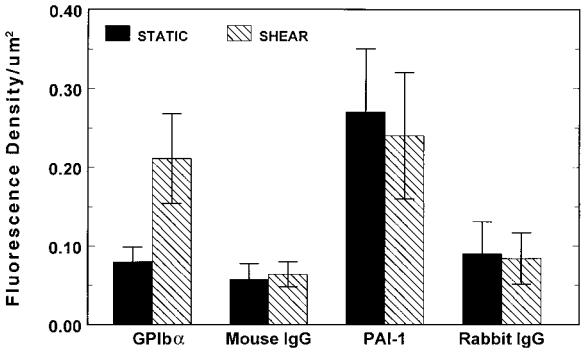


**Fig. 4.** Quantitation of cell surface immunofluorescence after 7 h of shear stress. Cell surface immunofluorescence of paraformaldehyde-fixed HUVEC was quantified in arbitrary units of density/μm2 with NIH 1.61b8 software on the Macintosh 7100/66 Image Analysis system. HUVEC treated under static conditions (solid bars) were compared with cells exposed to 30 dynes/cm$^2$ of shear stress for 7 h (cross-hatched bars). Endothelial cells were stained with anti-GpIbα Mab 1b1, nonimmune mouse IgG, rabbit anti PAI-1 or nonimmune rabbit IgG.

## DISCUSSION

The hemodynamic force of shear stress has been shown to modify endothelial cell structure and function, as manifested by changes in growth, morphology, gene transcription and protein synthesis, and secretion [Davies, 1989; Davies, 1995; Dewey et al., 1981; Gimbrone et al., 1997; Konstantopoulos and McIntire, 1996; Levesque and Nerem, 1985; Malek and Izumo, 1995; Resnick and Gimbrone, 1995; Malek and Izumo, 1994]. The signaling pathways mediating these responses are as yet poorly understood. Increases in $Ca^{2+}$, $IP_3$, and $PGI_2$ occur rapidly, within 2–5 min, but revert to baseline within 30–60 min, although the shear-induced increase in $PGI_2$ could be sustained for many hours if albumin-bound arachidonic acid was present in the culture medium [Frangos et al., 1985]. Other effects are somewhat longer lived: the increases in PDGF-B, TGF β-1, endothelin-1, and ICAM-1 gene expression occur within 1 h but drop to, or below, static levels within 6–8 h [Morigi et al., 1995; Nagel et al., 1994; Ohno et al., 1995; Resnick et al., 1993; Frangos et al., 1985; Morita et al., 1993]. Still other effects, such as increased tPA gene expression [Diamond et al., 1989], appear to be longer lasting, but only a few experiments have examined gene expression beyond 6–8 h of shear stress. Many of the effects cited may be important in the chain of signal transduction events leading to the steady-state response to shear stress, whereas other mechanisms may be required for maintaining that state.

A shear stress of 30 dynes/cm$^2$ was chosen for our experiments because it is in the middle to upper range for arterial blood flow. The two- to four-fold increases we observed in surface and total GP Ibα protein expression, is not unlike the induction of ICAM-1 (2.5-fold) [Nagel et al., 1994], tPA (two- to three-fold) [Diamond et al., 1989] , or TGFβ-1 (two-fold) [Ohno et al., 1995] protein expression in endothelial cells exposed to arterial shear stress for 24–48 h. The biotin EC surface labeling demonstrated a two- to four-fold increase in GP Ibα surface expression from 24–48 h. The GP Ibα increase on shear-treated EC we observed by flow cytometry after 48 h with the 1b1 mAb demonstrated two populations of HUVEC (Fig. 5) whereas the GC polyclonal antibody did not (Fig. 6). Since this two-peak effect was observed with at least one other anti-GP Ibα mAb (data not shown), it may be related to subtle changes in mAb binding

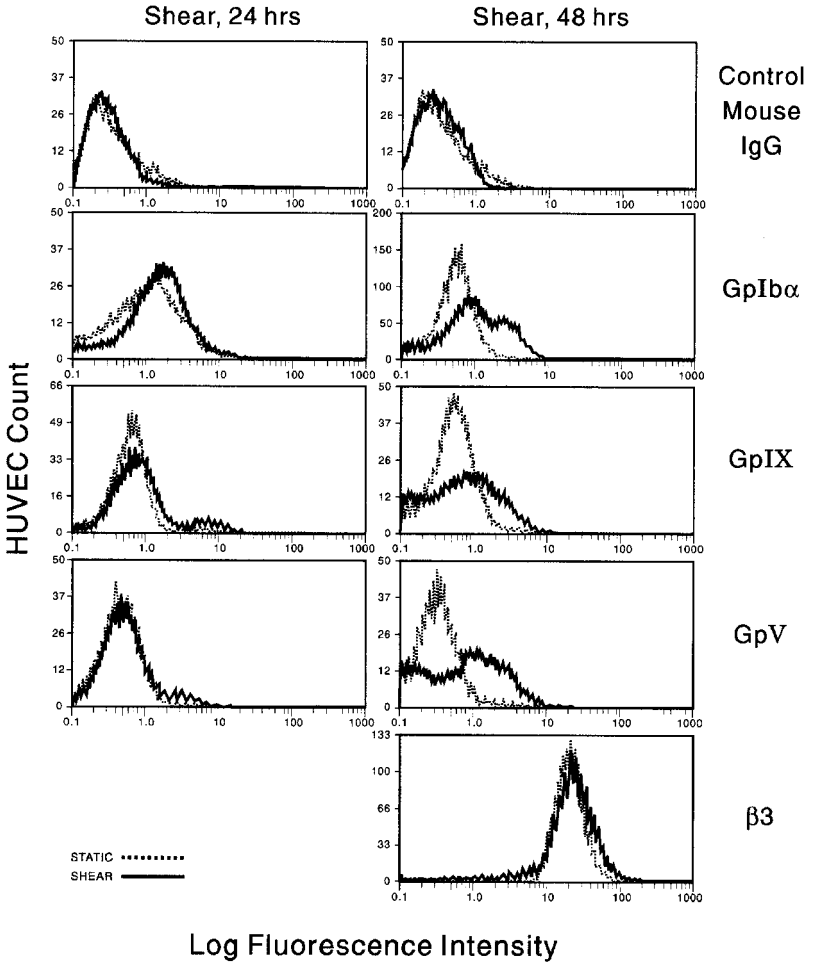


**Fig. 5.** Flow cytometric analysis of GpIbα, GpIX, and GpV after 24 and 48 h of shear stress. HUVEC were plated under static culture conditions or exposed to a shear stress of 30 dynes/cm$^2$ for 24 or 48 h. Identical numbers of static or sheared cells were stained with Mabs 1b1 (GpIbα), FMC-25 (GpIX), or 1D9 (GpV). Normal mouse IgG is shown in the top two panels. A dotted line denotes static HUVEC and a solid line represents shear-exposed HUVEC.

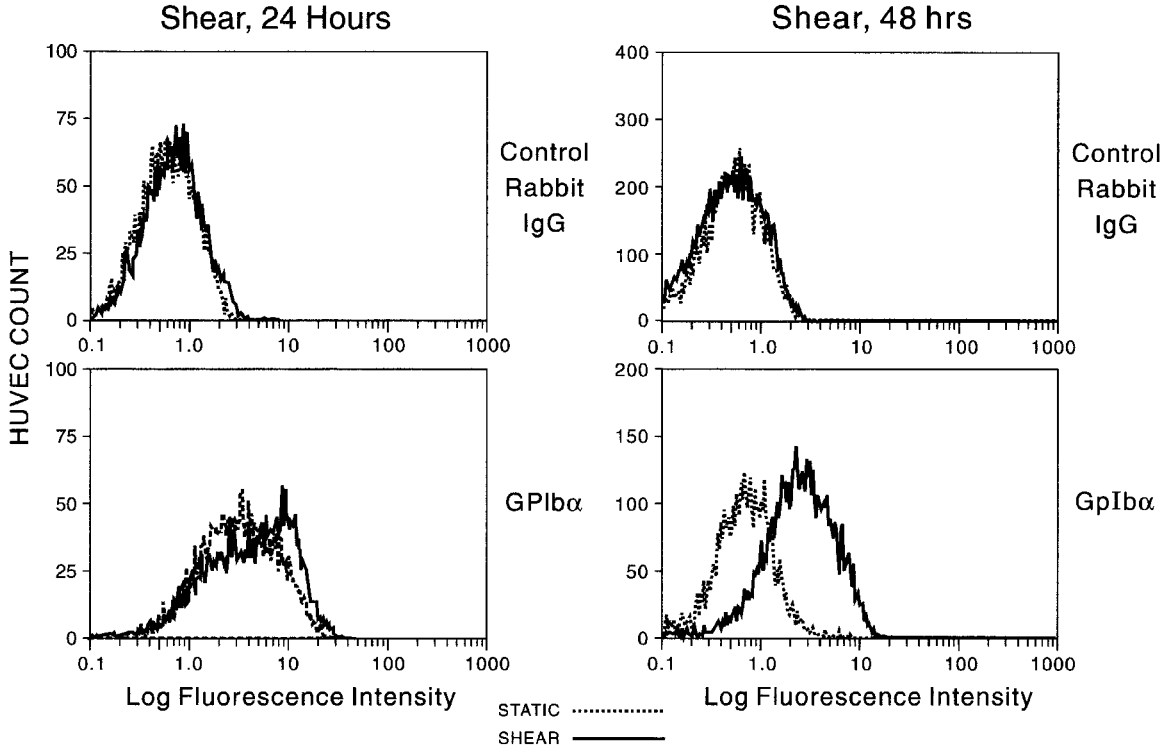


**Fig. 6.** Flow cytometric analysis of GpIbα with the polyclonal antibody to GC after 24 and 48 h of shear stress. Static and shear-stress treated HUVEC were treated with either a polyclonal rabbit antibody to glycocalicin or with normal rabbit IgG (control).

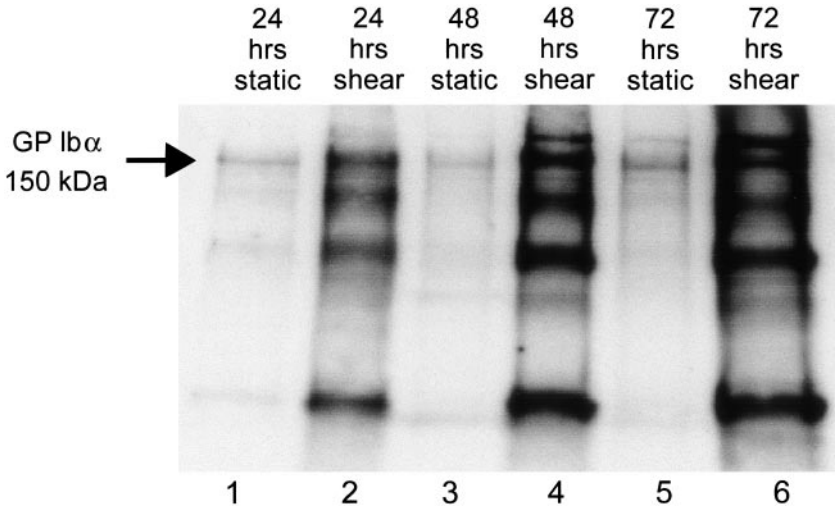


**Fig. 7.** Western blot analysis of surface biotinylated HUVEC proteins under static and shear conditions. Shear and static HUVEC were biotinylated and immunoprecipitated GP Ibα was detected with avidin-HRP as described. Static and shear samples are shown for 24 h (**lane 1**, static, and **lane 2**, shear), 48 h (**lane 3**, static and **lane 4**, shear) and 72 h (**lane 5**, static, and **lane 6**, shear).

which occur after HUVEC are exposed to prolonged shear stress. Differences in mAb binding at the 48 h time period imply that GP Ibα undergoes a conformational change over an extended exposure to shear stress, which is manifested by reduced mAb binding to a subpopulation of EC. However, the inability of the polyclonal Ab to demonstrate this bimodal effect suggests that comparable amounts of GP Ibα are on the surface of the entire HUVEC population. In summary, when our results are taken together they indicate that both total and cell surface expression of GP Ibα on HUVEC exposed to an arterial rate of shear stress was increased from 7–72 h, compared with static EC, with the highest levels of expression observed at 48 h.

Studies by Gimbrone and coworkers have implicated a consensus SSRE—the 5'-GAGACC-3' binding motif—in the response to shear. Although definitive evidence supporting GAGACC as the primary transcriptional regulator of shear stress has been adduced only for PDGF-B [Gimbrone et al., 1997; Knachigian et al., 1995; Resnick et al., 1993]. and, with less certainty, for ICAM-1 [Nagel et al., 1994], the SSRE sequence has been identified in the promoter region of a number of genes whose products are known to be increased by shear stress. Thus, the GAGACC SSRE appears to represent an important common theme in shear-responsive genes [Gimbrone et al., 1997; Resnick and Gimbrone, 1995; Frangos et al., 1985]. However, not all shear-responsive genes may operate by this mechanism. For example, although the MCP-1 gene contains two SSREs in its promoter region, a TRE site, an AP-1 family member, has been implicated in the shear-induced transcriptional activation of this gene [Resnick and Gimbrone, 1995]. Moreover, other promoter elements, including a negative shear-responsive element in the preproendothelin-1 gene identified by Malek and coworkers, may down-regulate certain endothelial genes [Malek and Izumo, 1994]. The GpIbα gene [Wenger et al., 1988] contains seven copies of the GAGACC sequence or its complementary sequence, GGTCTC, between positions -1314 and -125 5' to the GpIbα transcription start site [Hashimoto and Ware, 1995] and, as we have shown, GpIbα is upregulated by shear stress. GP Ibα mRNA expression was also increased over a 3–48 h period of exposure to shear stress, as measured by RT-PCR analysis (data not shown). In contrast with these results, RT-PCR analysis indicated that neither the mRNA expression of PAI-1, evaluated after 5 h of flow, nor the small ribosomal protein S14 [Leonard et al., 1993], analyzed for 0–48 h of shear exposure, was affected by a shear stress of 30 dynes/cm$^2$ (data not shown).

The mechanism of shear-induced up-regulation of the GpIb complex on the EC surface is not known. López and coworkers have demon-

strated that efficient display of transfected GpIbα on the surface of Chinese hamster ovary (CHO) cells requires the concomitant presence of GpIbβ and GpIX cDNA [López et al., 1992]. Since GpIbα mRNA concentration is very low in nonstimulated EC, it is possible that the effect of shear on the entire complex is mediated through up-regulation of GpIbα mRNA. This possibility is strengthened by the lack of SSRE sequences in the promoter regions of GpIbβ and GpIX, although the existence of other shear modulatory elements [Resnick and Gimbrone, 1995; Malek and Izumo, 1994] indicates that the effect of shear on Gp Ibα, GpIX, and GpV mRNA needs to be determined directly. Although GpV contains an SSRE sequence within its promoter [Lanza et al., 1993], the contribution of GpV to surface expression of the GpIb complex is probably minor [Li et al., 1995].

In our studies, both the EC actin cytoskeleton and overall EC morphology realigned parallel to the direction of flow after 48 h of shear exposure, resembling the *in vivo* phenotype of the endothelium [Davies, 1989; Davies, 1995; Dewey et al., 1981; Levesque and Nerem, 1985]. Thus, the sustained increase in GpIbα surface expression suggests that GpIbα may be present on the endothelial surface *in vivo* in greater amounts than implied by studies of static cultures. Since exposure of EC for 24 h to a shear stress of 3 dynes/cm$^2$, in the range of values seen in venous blood, did not appreciably up-regulate GpIbα mRNA, it is possible that surface expression of the EC GpIb complex *in vivo* is lower in veins than in arteries. Experiments to evaluate GP Ibα expression in the arterial and venous vasculature *in vivo* are currently in progress. Although the *in vivo* role of the EC GpIb complex has yet to be defined, it is capable of binding vWF [Asch et al., 1988], and can mediate EC adhesion to vWF under static conditions [Beacham et al., 1995; Beacham et al., 1997; Beacham et al., 1992], particularly when expression of the vitronectin receptor is downregulated and GpIbα is up-regulated as occurs in the presence of TNF-α and IFN-γ [Beacham et al., 1997; Wu et al., 1995; Defilippi et al., 1991]. Moreover, in recent studies, we have implicated endothelial GP Ibα in HUVEC migration on vWF [Beacham et al., 1996]. Thus, the EC GpIbα complex may be one of several EC receptors which mediate EC-matrix interactions during wound healing or angiogenesis [Dejana et al., 1989; Dejana et al., 1988]. Future studies of endothelial GpIbα expression and function under steady-state shear stress may provide further insight into its role in vascular homeostasis.

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